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ORIGINAL ARTICLE

MiR-5571-3p and miR-135b-5p, derived from analyses of microRNA profile sequencing, correlate with increased disease risk and activity of rheumatoid arthritis

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Abstract

Objectives This study aimed to investigate microRNA (miRNA) expression profiles in synovium tissues of rheumatoid arthritis (RA) patients by RNA sequencing and to evaluate the values of dysregulated miRNAs in RA diagnosis and monitoring.

Methods Thirty RA patients who underwent knee arthroscopy and 30 controls with knee trauma who underwent surgery were consecutively recruited, and synovium tissue samples of both groups were obtained during surgeries. In the exploration part, miRNA and mRNA expression profiles of 3 RA samples and 3 control samples were detected using RNA sequencing then followed by bioinformatic analyses. In the validation part, 5 candidate miRNA levels were detected by quantitative polymerase chain reaction (qPCR) in 30 RA patients and 30 control patients.

Results In the exploration part, 78 miRNAs and 1582 mRNAs were upregulated while 40 miRNAs and 1295 mRNAs were downregulated in synovium tissue samples of RA patients compared with those of controls. Furthermore, enrichment analyses revealed that these dysregulated miRNAs and mRNAs were mainly implicated in immune activities and inflammatory diseases such as leukocyte migration, complement activation, and RA. In the validation part, qPCR assay revealed that miR-5571-3p and miR-135b-5p expressions were increased in RA patients compared with those in controls and disclosed good predictive values for RA risk with high area under the curves (AUCs). Besides, both miR-5571-3p and miR-135b-5p levels were positively correlated with disease activity and inflammation level of RA.

Conclusions Analyses of miRNA expression profiles by sequencing indicate that miR-5571-3p and miR-135b-5p correlate with increased RA risk and activity.

Keywords Expression profiles · MicroRNA · Rheumatoid arthritis · RNA sequencing · Synovium tissue

Introduction

Rheumatoid arthritis (RA), one of the most common autoimmune diseases, is characterized by chronic synovitis, joint lesions, and other extra-articular comorbidities such as cardiovascular disease [1, 2]. RA affects 0.42% of the Chinese population and 1% of the world population and could cause lifelong disability if RA patients are treated inappropriately [1, 2]. Current therapeutic drugs including disease-modifying anti-rheumatic drugs as well as biological agents are effective in decreasing disease activity and improving quality of life for majority of RA patients, whereas there still lacks of sensitive and specific biomarker for RA monitoring, and most importantly, the pathogenesis of RA has not been fully illuminated, which greatly hinders the discovery of curable therapeutics [3–5].

Cailong Liu and Axiao Pan contributed equally to this work.

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MicroRNAs (miRNAs), a group of small, non-coding, and single-stranded RNAs that are widely distributed in viruses, animals, and human beings, play important roles in a number of biological activities through post-transcriptional regulation [6–9]. Previous studies suggest that miRNA expression profiles are implicated in different autoimmune diseases, including systemic lupus erythematosus (SLE), psoriasis, and inflammatory bowel disease (IBD), whereas for miRNA expression profiles in RA, only a few studies have been reported [10–14]. For instance, a study that investigates the miRNA expression profiles in plasma of RA patients using microarray identifies 22 upregulated and 11 downregulated miRNAs in RA patients compared with healthy controls (HCs), and further analyses discover 9 miRNAs that could be served as biomarkers for RA diagnosis or monitoring [14]. Another small-sample study explores the miRNA expression profiles in peripheral blood mononuclear cells (PBMCs) of RA patients using microarray, which discloses 9 upregulated miRNAs and 5 downregulated miRNAs in RA patients compared to HCs, and further analyses indicate that miR-155 might be implicated in RA etiology via regulating its target mRNA (IKBKE) [15].

However, the majority of samples in these studies are obtained from peripheral blood (plasma or PBMCs), while miRNA expression profiles in peripheral blood are easily affected by many other factors which may not accurately disclose the etiology of RA. Besides, most detections are conducted by microarray rather than RNA sequencing, which means that they are less likely to spot novel transcripts; more importantly, rare bioinformatic analyses are applied in these reported studies [16]. As a consequence, the miRNA expression profiles of RA and their potential roles in RA development and progression have not been comprehensively investigated. Therefore, the objective of current study was to investigate miRNA expression profiles in synovium tissues of RA patients using RNA sequencing and to further evaluate the role of dysregulated miRNAs in RA pathogenesis via bioinformatic analysis as well as the clinical values of candidate miRNAs in RA diagnosis and monitoring.

Materials and methods

Patients and sample collection

Thirty RA patients who underwent knee arthroscopy and 30 controls with knee trauma underwent surgery between July 1, 2016, and June 30, 2018, at The First Affiliated Hospital of Wenzhou Medical University were consecutively enrolled in this case-control study. Synovium samples were obtained during knee arthroscopy or trauma surgery and then stored in liquid nitrogen for further detection. The inclusion criteria of RA patients were as follows: (1) diagnosis of RA according to 1987 American College of Rheumatology (ACR) classification of RA, (2) age above 18 years, (3) severe knee symptoms underwent knee arthroscopy, and (4) without a history of knee surgery. The inclusion criteria of controls were as follows: (1) trauma of knee joint underwent surgery, (2) without a history of knee degeneration disease or inflammatory diseases, and (3) without a history of knee surgery.

Study flow

This study was divided into two major parts: exploration part and validation part. In the exploration part, 3 synovium tissue samples from RA patients and 3 synovium tissue samples from controls were applied for RNA sequencing to detect the miRNA expression profiles and mRNA expression profiles. In the validation part, 30 synovium tissue samples from RA patients and 30 synovium tissue samples from controls were applied for quantitative polymerase chain reaction (qPCR) to detect expressions of candidate miRNAs derived from RNA sequencing.

Ethics approval

This study was approved by the Ethics Review Board of The First Affiliated Hospital of Wenzhou Medical University, and written informed consents of all participants were obtained before enrollment.

Collection of RA patients' clinical data

After enrollment, clinical data of RA patients were collected, which included the following: age, gender, body mass index (BMI), disease duration, tender joint count (TJC), swollen joint count (SJC); erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and disease activity score in 28 joints (DAS28), rheumatoid factor (RF) status, anti-citrullinated protein antibodies (ACPA) status.

Library generation and sequencing

After the extraction of total RNA from 3 synovium tissue samples of RA patients and 3 synovium tissue samples of controls using TRIzol reagent (Invitrogen, USA), the concentration (>=500 ng/μl is qualified), purity (OD260/OD280 range from 1.8 to 2.2), and integrity (28S:18S>=1.5) were detected using Qubit® RNA Assay Kit with Qubit® 2.0 Fluorometer (Life Technologies, USA), NanoPhotometer® spectrophotometer (IMPLEN, USA), and RNA Nano 6000 Assay Kit with Bioanalyzer 2100 system (Agilent Technologies, USA), respectively. mRNA was captured by Dynabeads Oligo $(dT)_{25}$ (Thermo Fisher Scientific), while miRNA was captured by mirvana™ miRNA isolation kit (Thermo Fisher Scientific). Subsequently, first and second

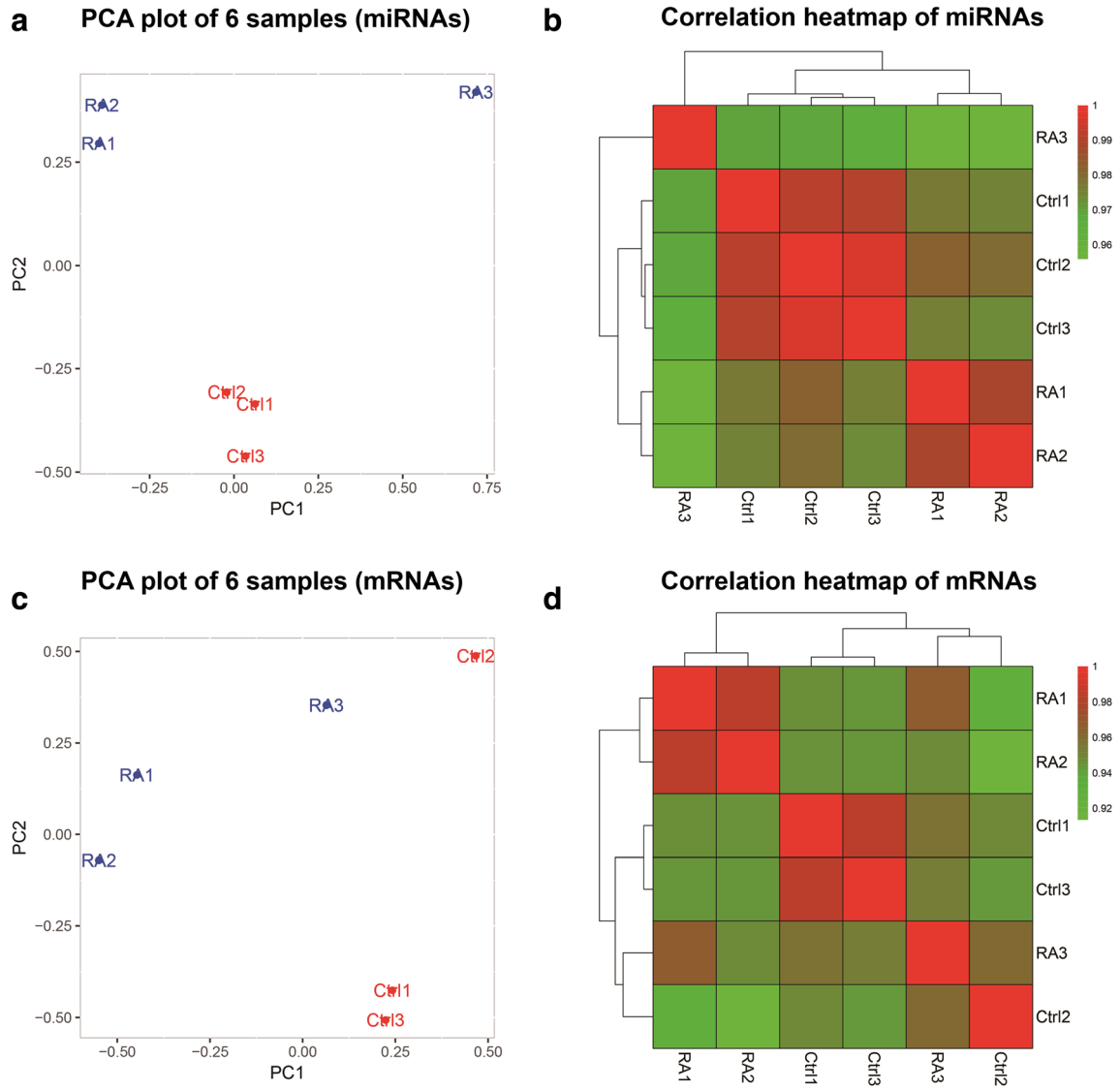

Fig. 1 PCA analysis and correlation heatmap analysis. miRNA expression profiles of 6 samples could differentiate RA patients from controls (**a**). RA patients and controls were separately clustered together by miRNA expression profiles of 6 samples, except for RA3 and Ctrl2 (**b**). mRNA expression profiles of 6 samples were also able to differentiate RA patients from controls (**c**). RA patients and controls were separately clustered together by mRNA expression profiles of 6 samples, except for RA3 and Ctrl2 (**d**). PCA and heatmap plot were performed by stats and pheatmap packages. PCA, principal component analysis; miRNA, microRNA; RA, rheumatoid arthritis

strands of the cDNA were synthesized, and library fragments were purified using AMPure XP system (Beckman Coulter, USA). PCR assay was then conducted followed by the measurement of quality of the library using Bioanalyzer 2100 system (Agilent Technologies, USA). Finally, clustering of index-coded samples was then performed using HiSeq PE Cluster Kit v4 cBot (Illumina, USA), and the libraries were sequenced on Illumina HiSeq X10 platform (Illumina, USA) and 150-bp paired-end reads and 50-bp single-end reads were produced after cluster generation for mRNA and miRNA, respectively.

Workflow of RNA sequencing

Automate quality control and adapter trimming were conducted using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), Cutadapt (http://cutadapt.readthedocs.io/en/stable/), and FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Subsequently, the trimmed reads were mapped to the human genome Hg38 by Hisat2 (mRNA) and Bowtie (miRNA) with the default parameters referring to a previous report [17], and mapping quality control including library generation method detection and read mapping efficiency was conducted using RSeQC referring to

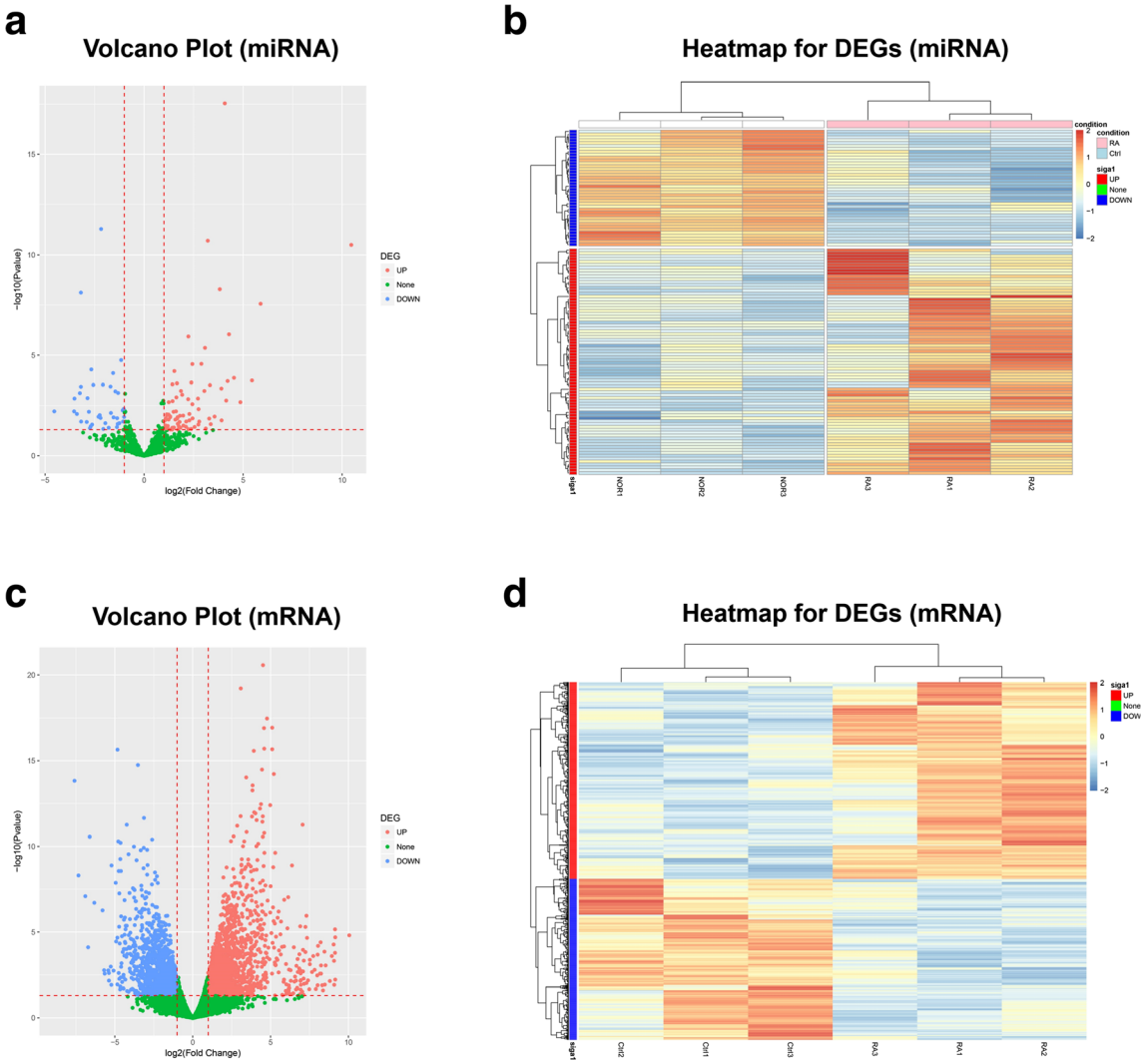

Fig. 2 Volcano plot and heatmap analysis. Seventy-eight miRNAs were upregulated while 40 miRNAs were downregulated in RA patients compared to those in controls (**a**). DEGs (miRNAs) well differentiated RA patients from controls (**b**). As to DEG analyses for mRNA, 1582 were upregulated whereas 1295 were downregulated in RA patients compared with those in controls (**c**). RA patients and controls were also well differentiated by DEGs (mRNAs) (**d**). The blue color represented downregulated genes, and the red color represented upregulated genes while the green color represented not differentially expressed genes. DEGs were determined by DeSeq2, and clinical significance was defined as a fold change ≥ 2.0 and *P* value < 0.05. miRNAs, microRNAs; RA, rheumatoid arthritis; DEGs, differentially expressed genes

another previous paper [18]. The read counts of miRNA and mRNA were then calculated using featureCounts according to a previous report [19] based on miRBase V21 (http://www.mirbase.org) and the annotation file (Homo_sapiens.GRCh38.83.gtf) in Ensemble database. The miRNAs and mRNAs discovered in 50% or above samples were retained for analysis. Subsequently, the raw read counts were normalized, logarithmic transformation was applied, and differentially expressed genes (DEGs) were detected using DeSeq2 as previously described [20]. The statistical significance was defined as *P* value < 0.05, and the biological significance was defined as a difference of at least 2.0 folds, abs ($\log_2$ (fold change)) > 1.0.

Bioinformatics analysis

The bioinformatics analysis was performed mainly using R software (Version 3.3.3) as follows: (1) principal component analysis (PCA) and heatmap plot of expression pattern were completed by stats and pheatmap packages; (2) based on the targets of miRNAs predicted by miRanda and annotation of mRNA in Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database, enrichment analysis of DEGs (mRNA and miRNA) was performed using DAVID web servers according to a previous report [21]; (3) regulatory network of top 10 upregulated and 10 downregulated DEGs (miRNA) was drawn using igraph package; (4) circos graph

Table 1 Top 10 upregulated and 10 downregulated miRNAs in RA synovium

Gene symbol	LogFC	P value	Trend
hsa-miR-5571-3p	10.46509	3.15E−11	Up
hsa-miR-135b-5p	5.884883	2.71E−08	Up
hsa-miR-31-3p	5.447289	0.000178	Up
hsa-miR-7976	4.864507	0.002122	Up
hsa-miR-138-5p	4.541739	0.000133	Up
hsa-miR-31-5p	4.290969	9.02E−07	Up
hsa-miR-2682-5p	4.217122	0.000194	Up
hsa-miR-1295a	4.150197	0.001767	Up
hsa-miR-182-5p	4.075572	2.86E−18	Up
hsa-miR-548ba	3.908272	0.017084	Up
hsa-miR-190a-3p	−4.53203	0.00608	Down
hsa-miR-196b-3p	−3.53219	0.006157	Down
hsa-miR-676-3p	−3.52018	0.001414	Down
hsa-miR-516a-5p	−3.39168	0.008265	Down
hsa-miR-216a-5p	−3.24721	0.000759	Down
hsa-miR-653-3p	−3.1921	7.55E−09	Down
hsa-miR-516b-5p	−3.18768	0.000367	Down
hsa-miR-548aw	−3.17954	0.01958	Down
hsa-miR-561-5p	−2.89508	0.020705	Down
hsa-miR-1248	−2.85375	0.001358	Down

miRNA, microRNA; *RA*, rheumatoid arthritis; *FC*, fold change

for transcription and regulation information was drawn using RCircos package as described in a previous report [22].

Selection of candidate miRNAs

To further explore the role of top DEGs (miRNA) in RA, 3 upregulated miRNAs (miR-5571-3p, miR-135b-5p, and miR-31-3p) and 2 downregulated miRNAs (miR-190a-3p and miR-196b-3p) were selected, and their expressions were further detected by qPCR in 30 RA patients and 30 controls.

qPCR assay

Total RNA was extracted from 30 synovium tissue samples of RA patients and 30 synovium tissue samples of controls using TRIzol reagent (Invitrogen, USA), and the concentration, purity, and integrity control were conducted by NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). Then cDNAs were synthesized with QuantiTect Rev. Transcription Kit (Qiagen, German), and subsequently subjected to qPCR with SYBR Green kit (TaKaRa, Japan). The PCR amplification procedures were as follows: degeneration at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s, then 60 s at 60 °C. After that, the expressions of 5 candidate miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ methods with U6 as an internal reference. Detailed primers used in qPCR assay were listed in Supplementary Table 1.

Statistics

Statistical analysis was performed by the SPSS 21.0 software (IBM Corp, USA) and GraphPad Prism 6.01 (GraphPad Software Inc., USA). Data were presented as mean value ± standard deviation, median value (range), or count (percentage). Comparison between two groups was determined by the Wilcoxon rank-sum test; receiver operating characteristic (ROC) curve was drawn to assess the ability of miRNA expressions to distinguish RA patients from controls; the Spearman test was used to evaluate the association of miRNA expressions with continuous variables in RA patients. $P < 0.05$ was considered significant.

Results

PCA analysis and correlation heatmap analysis for total miRNA and mRNA patterns

PCA plot of miRNAs revealed that miRNA expression profiles of 6 samples clearly differentiated RA patients from controls (Fig. 1a), and correlation heatmap analysis showed that RA patients and controls were separately clustered together by miRNA expression profiles of 6 samples, except for RA3 and Ctrl2 (Fig. 1b). Meanwhile, PCA plot of mRNAs disclosed that mRNA expression profiles of 6 samples were also able to differentiate RA patients from controls (Fig. 1c), and correlation heatmap analysis disclosed that RA patients and controls were separately clustered together by mRNA expression profiles of 6 samples, except for RA3 and Ctrl2 (Fig. 1d). The possible explanation for not well differentiating RA3 from Ctrl2 might be that RA3 might have several extremely similar characteristics with Ctrl2, such as age, gender, and BMI.

Volcano plot and heatmap analysis for DEGs (both miRNA and mRNA)

Volcano plot for miRNA showed that 78 miRNAs were upregulated while 40 miRNAs were downregulated in RA patients compared with those in controls (fold change ≥ 2.0 and P value < 0.05) (Fig. 2a), and the top 10 upregulated and the top 10 downregulated miRNAs were displayed in Table 1. Heatmap analysis for DEGs (miRNA) revealed that these DEGs (miRNAs) well differentiated RA patients from controls (Fig. 2b). As to DEG analyses for mRNA, 1582 were upregulated whereas 1295 were downregulated in RA patients compared to those in controls (Fig. 2c), and heatmap analysis disclosed that RA patients and controls were also well distinguished by DEGs (mRNAs) (Fig. 2d).

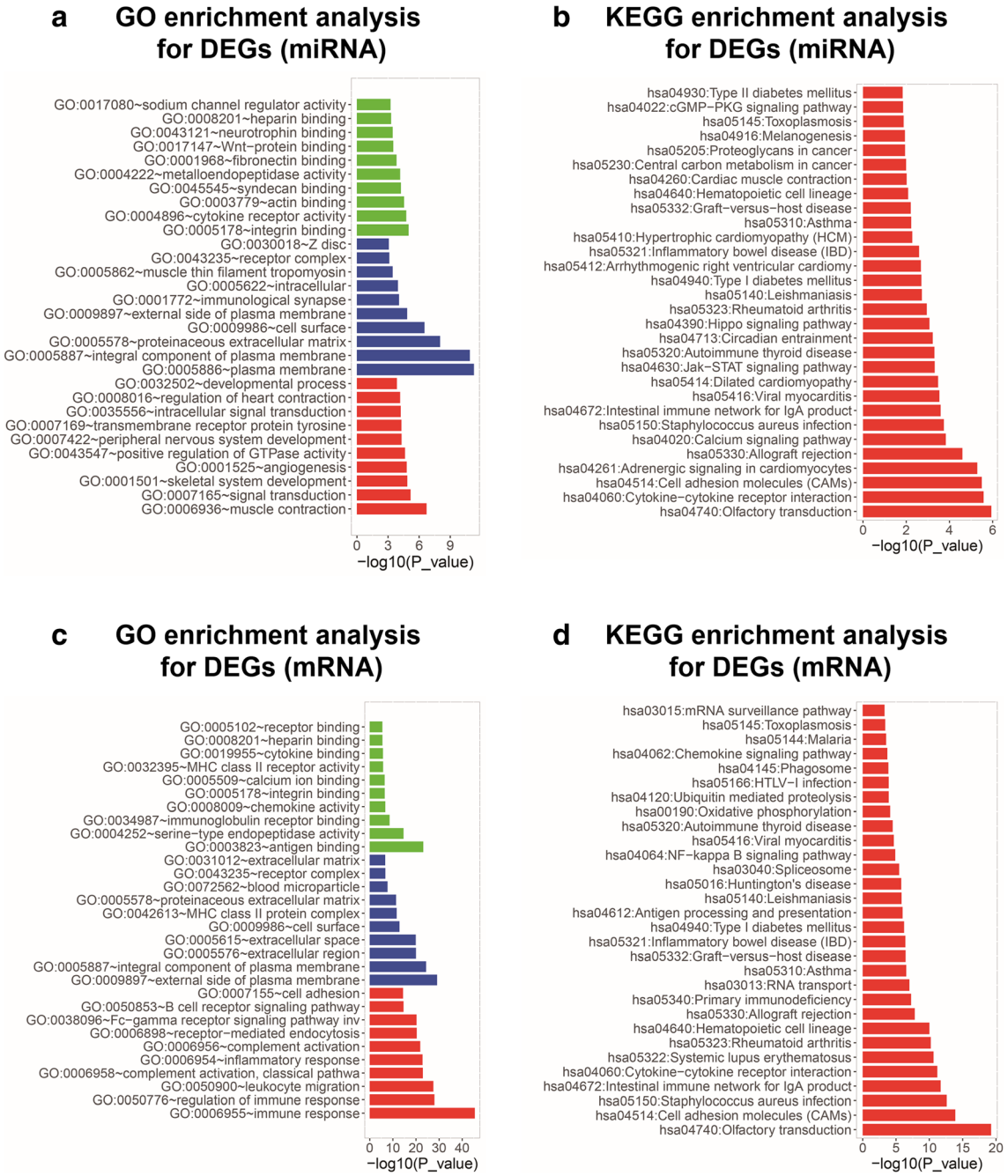

Fig. 3 GO and KEGG enrichment analyses for DEGs (miRNAs and mRNAs). GO enrichment analysis revealed that DEGs (miRNA) were associated with a variety of cellular components and biological processes, such as plasma membrane, integral component of plasma membrane, and muscle contraction (**a**). KEGG enrichment analysis showed that DEGs (miRNA) were correlated with different pathways, including olfactory transduction, cytokine-cytokine receptor interaction, and intestinal immune network for IgA product (**b**). GO enrichment analysis disclosed that DEGs (mRNA) were most implicated in immune activities such as immune response, regulation of immune response, leukocyte migration, and complement activation (**c**). KEGG enrichment analysis revealed that DEGs (mRNA) were involved in various immune diseases, including RA, SLE, and primary immunodeficiency (**d**). Enrichment analyses for targets of DEGs were performed using DAVID web servers. GO, Gene Ontology; KEGG, Kyoko Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; miRNA, microRNA; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus

GO and KEGG enrichment analyses

In order to investigate the biological activities and pathways that these DEGs might be implicated in, GO and KEGG enrichment analyses were performed. GO enrichment analysis showed that DEGs (miRNA) were correlated with a variety of cellular components and biological processes such as plasma membrane, integral component

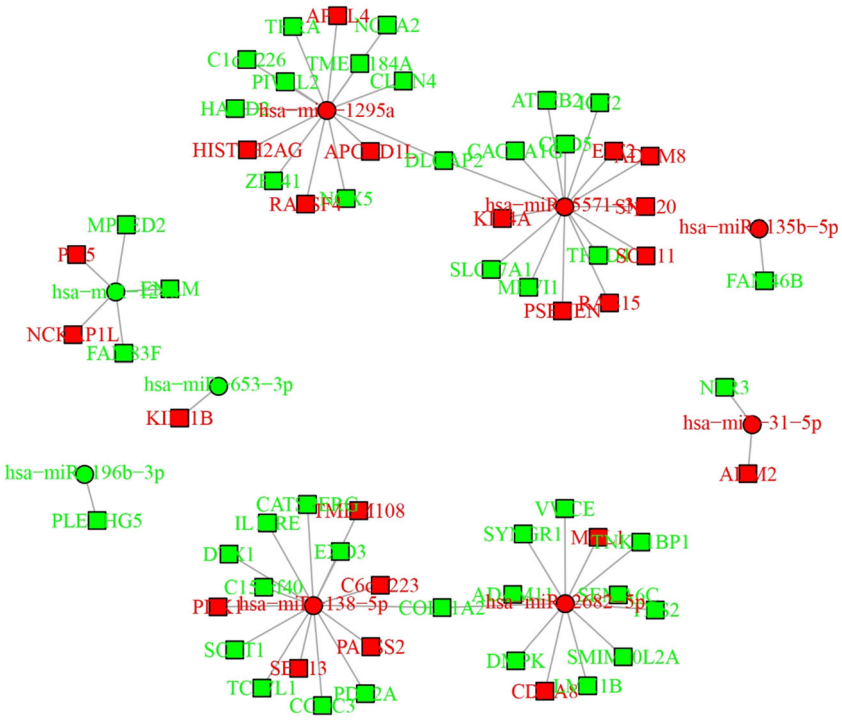

Fig. 4 Regulatory network for DEGs. The interactions between miRNAs and their target mRNAs were displayed. The round point represented candidate miRNAs, while the squared point represented targeted mRNAs. Red color meant upregulated genes while green color meant downregulated genes. Regulatory networks of all DEGs were drawn using igraph package. DEGs, differentially expressed genes; miRNAs, microRNAs

of plasma membrane, and muscle contraction (Fig. 3a); KEGG enrichment analysis revealed that DEGs (miRNA) were implicated in different pathways, including olfactory transduction, cytokine-cytokine receptor interaction, and intestinal immune network for IgA product (Fig. 3b). As to enrichment analyses for DEGs (mRNA), GO enrichment analysis illuminated that DEGs (mRNA) were mostly associated with immune activities such as immune response, regulation of immune response, leukocyte migration, and complement activation (Fig. 3c). KEGG enrichment analysis showed that DEGs (mRNA) were linked to various immune diseases, including RA, systemic lupus erythematosus (SLE), and primary immunodeficiency (Fig. 3d). These enrichment analyses indicated that DEGs (miRNA and mRNA) were involved in inflammatory activities and immune disease developments, including RA pathogenesis.

Regulatory network

In order to further explore the regulations of DEGs (miRNAs) with their target mRNAs in RA patients, the regulatory network was drawn in the current study. Considering that the whole regulatory network was complicated, which was not easy to distinguish, we only displayed the top 10 upregulated and top 10 downregulated miRNAs, among which 11 miRNAs had no target mRNAs; thus, only 9 DEGs (miRNA) and their target mRNAs were displayed in the regulatory network (Fig. 4).

Circos graph

Circos graph of transcription and regulation information was drawn with RCircos package to comprehensively present the roles of miRNA and mRNA expression profiles in pathogenesis of RA and interactions between miRNAs and mRNAs, which were presented as follows: the outermost layer stood for the chromosome number, the second outermost layer represented DEGs (mRNA), the third outermost layer meant DEGs (miRNA), and the inner collected lines meant regulation network between miRNA and its target mRNAs. In the graph, red color meant upregulated DEGs, green color referred to downregulated DEGs, and the height of column meant $\log_2$(fold change) **(**Fig. 5**)**.

Features of RA patients in validation part

The characteristics of 30 RA patients in validation part were displayed in Table 2 as follows: mean age was 56.77 ± 12.70 years, and numbers of male and female were 6 (20.0%) and 24 (80.0%), respectively. TJC and SJC were 6 (3–13) joints and 6 (1–18) joints; meanwhile, ESR and CRP were 44.23 (5.73–79.56) mm/h and 60.12 (19.93–222.74) mg/l, respectively. As for DAS28 score (ESR) and DAS28 (CRP), the mean values were 5.26 ± 0.66 and 5.21 ± 0.58, respectively.

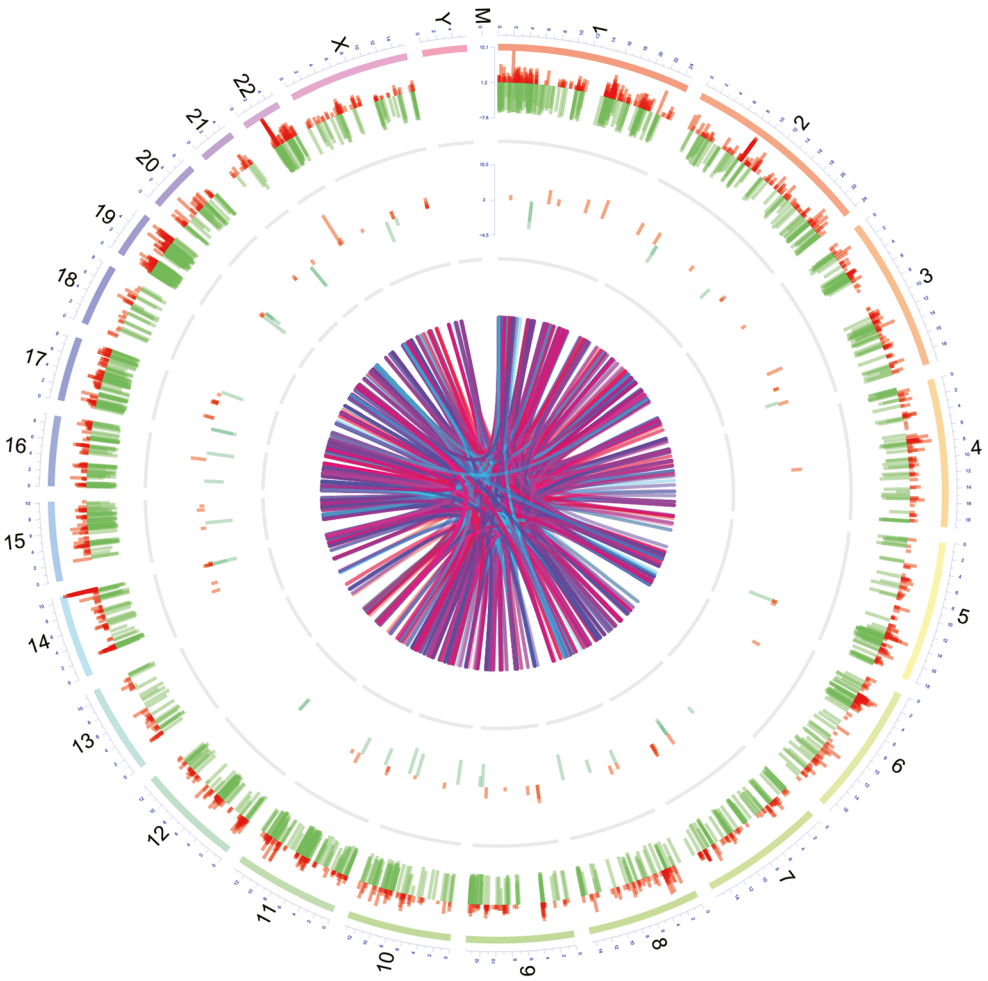

Fig. 5 Circos graph of transcription and regulation information. The outermost layer stood for chromosome number, the second outermost layer represented DEGs (mRNA), the third outermost layer meant DEGs (miRNA), and the inner collected lines stood for regulation network between miRNA and its target mRNAs. In the graph, red color meant upregulated DEGs, green color meant downregulated DEGs, and the height of column meant $\log_2$(fold change). Circos graph was drawn with RCircos package. DEGs, differentially expressed genes; miRNA, microRNA

Comparison of candidate miRNA levels between RA patients and controls in validation part

Among the 5 candidate miRNAs, miR-5571-3p ($P < 0.001$, Fig. 6a) and miR-135b-5p ($P = 0.003$, Fig. 6b) were upregulated in RA patients compared with those in controls, while miR-31-3p ($P = 0.447$, Fig. 6c), miR-190a-3p ($P = 0.073$, Fig. 6d), and miR-196b-3p ($P = 0.082$, Fig. 6e) levels between the two groups were similar.

ROC curve analyses

ROC curves were applied for analyzing values of miR-5571-3p and miR-135b-5p for RA risk, which disclosed that areas under the curves (AUCs) of miR-5571-3p and miR-135b-5p were 0.803 (95%CI 0.692–0.915) and 0.721 (95%CI 0.594–0.849), respectively, and when combining miR-5571-3p with miR-135b-5p, the AUC was up to 0.833 (95%CI 0.732–0.935) (Fig. 7). The ROC curve analyses indicated that miR-5571-3p, miR-135b-5p, and their combination disclosed good predictive values for RA risk.

Correlations of candidate miRNA expressions with characteristics of RA patients

The Wilcoxon rank-sum test or Spearman test was applied for analyzing correlations of candidate miRNA levels with characteristics of RA patients, which revealed that miRNA-5571-3p was positively associated with ESR ($P = 0.036$), CRP ($P = 0.029$), and DAS28 (ESR) ($P = 0.042$) and miRNA-135b-5p was positively associated with CRP ($P = 0.018$), while miR-190a-3p was negatively correlated with CRP ($P = 0.009$). In addition, no correlation of these 5 miRNA expressions with other characteristics was observed (all $P > 0.05$, Tables 3 and 4).

Discussion

In the current study, 78 miRNAs and 1582 mRNAs were upregulated while 40 miRNAs and 1295 mRNAs were downregulated in synovium tissue samples of RA patients compared to those of controls, and these dysregulated miRNAs

Table 2 Characteristics of RA patients in validation part

Parameters	RA patients ($N = 30$)
Age (years)	56.77 ± 12.70
Gender (*n*/%)	
Male	6 (20.0)
Female	24 (80.0)
BMI (kg/m^2)	22.61 ± 3.89
Disease duration (years)	9 (5–16)
RF positive (*n*/%)	24 (80.0)
ACPA positive (*n*/%)	23 (76.7)
TJC (joints)	6 (3–13)
SJC (joints)	6 (1–18)
ESR (mm/h)	44.23 (5.73–79.56)
CRP (mg/l)	60.12 (19.93–222.74)
DAS28 score (ESR)	5.26 ± 0.66
DAS28 score (CRP)	5.21 ± 0.58

Data were presented as mean value ± standard deviation, median value (range), or count (percentage)

RA, rheumatoid arthritis; *BMI*, body mass index; *RF*, rheumatoid factor; *ACPA*, anti-citrullinated protein antibody; *TJC*, tender joint count; *SJC*, swollen joint count; *ESR*, erythrocyte sedimentation rate; *CRP*, C-reactive protein; *DAS28*, disease activity score in 28 joints

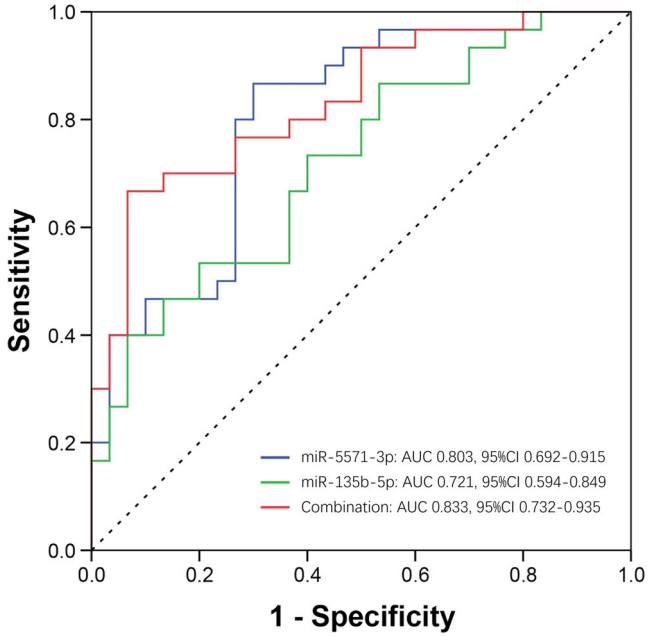

Fig. 7 ROC curve analyses for RA risk. ROC curves revealed that AUCs of miR-5571-3p and miR-135b-5p were 0.803 (95%CI 0.692–0.915) and 0.721 (95%CI 0.594–0.849), respectively; when combined miR-5571-3p with miR-135b-5p, the AUC was 0.833 (95%CI 0.732–0.935). ROC curves were drawn to evaluate the diagnostic values of miR-5571-3p and miR-135b-5p for RA risk. ROC, receiver operating characteristic; RA, rheumatoid arthritis; AUC, area under the curve; miRNA, microRNA

and mRNAs were associated with several immune activities and inflammatory diseases such as immune response, leukocyte migration, RA, and SLE. In validation part, miR-5571-3p and miR-135b-5p expressions elevated and disclosed good predictive values for RA risk with high AUCs. In addition, both miR-5571-3p and miR-135b-5p levels were positively correlated with disease activity and inflammation level of RA.

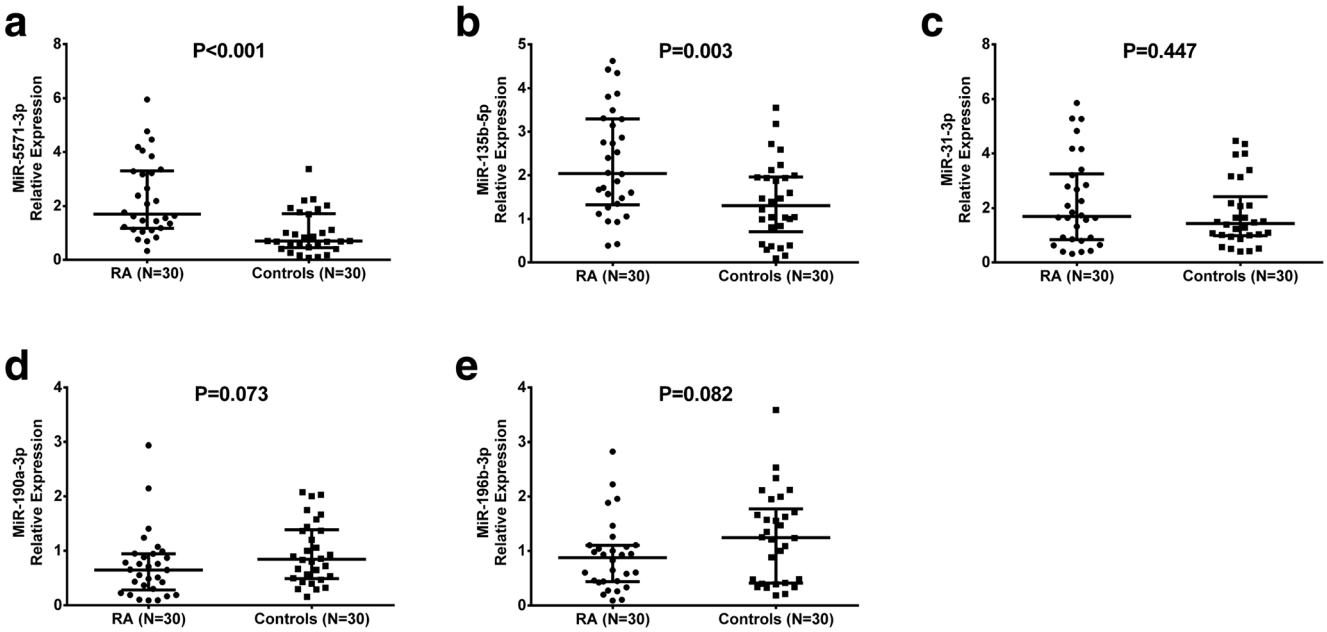

Fig. 6 Candidate miRNA expressions between RA patients and controls in validation part. miR-5571-3p (**a**) and miR-135b-5p (**b**) in RA patients were upregulated compared to those in controls, whereas miR-31-3p (**c**), miR-190a-3p (**d**), and miR-196b-3p (**e**) levels between two groups were of no difference. Comparison between two groups was determined by the Wilcoxon rank-sum test. $P < 0.05$ was considered significant. miRNA, microRNA; RA, rheumatoid arthritis

Table 3 Correlation of candidate miRNA expressions with patients' characteristics (discontinuous variables) in RA patients

	miR-5571-3p	miR-135b-5p	miR-31-3p	miR-190a-3p	miR-196b-3p
Gender					
Male	1.29 (0.83–4.77)	2.36 (1.06–4.43)	2.00 (0.40–5.29)	0.43 (0.17–0.99)	0.96 (0.11–2.22)
Female	2.13 (0.33–5.95)	2.04 (0.39–4.62)	1.70 (0.32–5.86)	0.73 (0.09–2.94)	0.72 (0.09–2.83)
P value	0.321	0.527	0.900	0.230	0.432
RF					
Positive	1.63 (0.33–5.95)	1.94 (0.39–4.62)	1.97 (0.32–5.86)	0.60 (0.09–2.94)	0.88 (0.20–2.22)
Negative	2.71 (0.76–4.77)	2.56 (1.57–3.31)	1.11 (0.39–2.67)	0.94 (0.17–1.41)	0.69 (0.09–2.83)
P value	0.374	0.631	0.057	0.347	0.595
ACPA					
Positive	1.64 (0.33–4.77)	2.03 (0.39–4.62)	1.84 (0.32–5.86)	0.66 (0.09–2.94)	0.65 (0.43–2.83)
Negative	2.08 (0.83–5.95)	2.40 (0.42–3.80)	1.64 (0.63–3.20)	0.65 (0.44–1.41)	0.93 (0.09–2.22)
P value	0.774	0.773	0.311	0.266	0.848

Data were presented as count (percentage). Comparison was determined by the Wilcoxon rank-sum test. $P < 0.05$ was considered significant

miRNA, microRNA; *RA*, rheumatoid arthritis; *RF*, rheumatoid factor; *ACPA*, anti-citrullinated protein antibody

Increasing evidences reveal that miRNA expression profiles play crucial roles in various autoimmune diseases such as IBD, SLE, and ankylosing spondylitis (AS) [23–25]. Nonetheless, for the roles of miRNA expression profiles in RA etiopathology, the detailed and comprehensive analysis is still limited. In a study conducted by Bellvitge Biomedical Research Institute, miRNA expression profiles in synovial fluids (SF) between RA patients and osteoarthritis (OA) patients are investigated using microarray, which identifies 60 dysregulated miRNAs in RA patients compared with OA patients, and further analysis indicates that these dysregulated miRNAs might be involved in RA pathogenesis via mediating a number of target genes, including CTSC, KLF8, EBF3, and NFAT5 [26]. In another study conducted in Peking University People's Hospital, miRNA expression profiles of PBMC in RA patients and osteoarthritis (OA) patients are analyzed using microarray, which reveals that 46 miRNAs are dysregulated in RA patients compared with those in OA patients [15]. However, the miRNA profiles in synovial tissue specimens of RA patients using RNA sequencing are rarely investigated, and the in-depth bioinformatic analyses of RA miRNA profiles such as enrichment analysis and regulatory network analysis are seldom conducted neither. Therefore, we conducted the current study that discovered 78 upregulated miRNAs and 40 downregulated miRNAs in synovium tissue of RA patients compared to controls. The numbers of dysregulated miRNAs in our study were numerically higher than in previously reported studies, which might be due to the following: firstly, RNA sequencing is capable of detecting more transcripts than microarray; thus, even though our study only included miRNAs that were detected in at least 50% samples, the number of dysregulated miRNAs in our study was still larger than in reported studies; secondly, the numbers of probes in microarray could be customized based on different needs, and in order to reduce the cost, these reported studies might only use a small number of probes in microarray to detect miRNAs, which cause relatively less dysregulated miRNAs; lastly, different controls (knee trauma patients vs OA patients), samples (synovium tissue samples vs PBMC), and research populations (Chinses vs Spanish) might also lead to discrepancies. As for enrichment analysis, it revealed that these dysregulated miRNAs and mRNAs were linked to a variety of immune activities and inflammatory diseases such as leukocyte migration, complement activation, RA, and SLE developments, which suggested that dysregulated miRNAs might be involved in RA pathogenesis via multiple ways, including leukocyte migration and complement activation. Additionally, regulatory network displayed the regulation functions of dysregulated miRNAs to their target mRNAs, which uncovered the detailed signaling pathways of RA that these dysregulated miRNAs might be implicated in. In brief, our study provided novel and comprehensive perspectives for understanding the association of miRNA expression profiles with pathogenesis of RA.

Since many miRNAs are observed to be involved in a variety of disease pathologies, investigation of these miRNAs might contribute to discovery of novel biomarkers for diagnosis and management of complicated diseases, including RA [27, 28]. According to several prior studies, both miR-155 and miR-146 are upregulated in RA patients compared with those in controls, and they are positively associated with disease activity [15, 29, 30]. In another study, 3 miRNAs (miR-4634, miR-181d, and miR-4764-5p) are observed to be upregulated while 6 miRNAs (miR-342-3p, miR-3926, miR-3925-3p, miR-122-3p, miR-9-5p, and miR-219-2-3p) are downregulated in RA patients compared with those in HCs;

Table 4 Correlation of candidate miRNA expressions with patients' characteristics (continuous variables) in RA patients

	miR-5571-3p	miR-135b-5p	miR-31-3p	miR-190a-3p	miR-196b-3p
Age					
Correlation coefficient *r*	0.144	− 0.155	0.063	− 0.215	0.269
P value	0.447	0.414	0.741	0.255	0.150
BMI					
Correlation coefficient *r*	0.051	0.198	0.064	0.261	0.145
P value	0.787	0.294	0.736	0.164	0.445
Disease duration					
Correlation coefficient *r*	0.012	− 0.176	− 0.062	0.058	− 0.104
P value	0.948	0.352	0.746	0.759	0.584
TJC					
Correlation coefficient *r*	0.116	− 0.094	− 0.228	0.113	− 0.166
P value	0.543	0.622	0.225	0.554	0.380
SJC					
Correlation coefficient *r*	0.198	− 0.074	− 0.027	0.160	− 0.120
P value	0.294	0.696	0.888	0.398	0.527
ESR					
Correlation coefficient *r*	0.385	0.277	− 0.006	− 0.244	0.152
P value	0.036	0.138	0.977	0.194	0.423
CRP					
Correlation coefficient *r*	0.398	0.427	− 0.244	− 0.471	0.154
P value	0.029	0.018	0.194	0.009	0.417
DAS28 score (ESR)					
Correlation coefficient *r*	0.373	0.137	− 0.198	− 0.143	− 0.021
P value	0.042	0.469	0.295	0.452	0.912
DAS28 score (CRP)					
Correlation coefficient *r*	0.302	0.176	− 0.314	− 0.152	− 0.064
P value	0.104	0.351	0.091	0.423	0.737

Data were presented as correlation coefficient *r* and *P* value. Correlation was determined by the Spearman test. $P < 0.05$ was considered significant

miRNA, microRNA; *RA*, rheumatoid arthritis; *BMI*, body mass index; *TJC*, tender joint count; *SJC*, swollen joint count; *ESR*, erythrocyte sedimentation rate; *CRP*, C-reactive protein; *DAS28*, disease activity score in 28 joints

meanwhile, all of the 9 miRNAs disclose good diagnostic values for RA with high AUCs [14]. These reported studies confirm that some miRNAs are dysregulated in RA patients and could serve as biomarkers for predicting RA risk or activity. However, there are still a great number of unelucidated miRNAs that might function as biomarkers for RA diagnosis or management. In order to discover these miRNAs, 3 upregulated and 2 downregulated miRNAs were selected from RNA sequencing analysis; then, their expressions were further validated by qPCR assay in synovium tissues of 30 RA patients and 30 controls. The results showed that miR-5571-3p and miR-135b-5p expressions were elevated in RA patients compared with those in controls and were positively associated with CRP, ESR, or DAS28 (ESR), indicating that miR-5571-3p and miR-135b-5p could be regarded as biomarkers for predicting RA risk and activity. The possible explanations might be that (1) miR-5571-3p is discovered in minor salivary glands of patients with Sjögren's syndrome (an autoimmune disease), implying that it might be correlated with inflammatory and immune activities of Sjögren's syndrome. Therefore, miR-5571-3p might also be implicated in inflammatory activity and immunobiology of RA. Meanwhile, the regulatory network in our study revealed that miR-5571-3p might regulate expressions of their target mRNAs (including THSD4 and CHD5) thereby mediated aberrant inflammatory and immune activities of RA [31]. (2) According to a previous study, IL-1α induces miR-135b expression in both NIH3T3 and FE1 cells within 60 min, implying that miR-135b-5p was involved in inflammation-related signaling pathways; hence, miR-135b-5p might also be associated with RA inflammatory activity. Moreover, recent studies suggest that miR-135b-5p promotes tumorigenesis in several cancers such as breast cancer, osteosarcoma, and hepatocellular carcinoma; thus, miR-135b-5p might also enhance abnormal proliferation of synoviocytes, thereby promote development and progression of RA. Lastly, miR-135b-5p implication in RA pathology might also

be via mediating its target mRNA FAM46B (as depicted in regulatory network) [32–35]. Therefore, both miR-5571-3p and miR-135b-5p expressions were increased in RA patients and could serve as biomarkers for RA risk and activity. As far as we know, this was the first study to discover that both miR-5571-3p and miR-135b-5p could be regarded as biomarkers for predicting RA risk and activity, which contributed to better understanding about RA etiology and investigating curable treatment agents.

There were some limitations in this study. Firstly, the study did not evaluate the treatment response of RA patients. Thus, the role of RA miRNA expression profiles in treatment response of RA patients remained unexplored. Secondly, the sample sizes of both RA patients and controls were relatively small, with only 30 RA patients and 30 controls; while considering that the synovium tissue samples were difficult to obtain, especially for controls, it was justified for the relatively small samples. At last, the majority of patients in the current study were from East China, which might bring in regional selection bias.

In summary, miRNA expression profiles play important roles in RA etiopathology, and both miR-5571-3p and miR-135b-5p might be served as novel biomarkers for predicting RA risk as well as activity.

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Compliance with ethical standards

This study was approved by the Ethics Review Board of The First Affiliated Hospital of Wenzhou Medical University, and all participants provided written informed consents before enrollment.

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